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Cytokine gene expression in ileal tissues of cattle infected with *Mycobacterium paratuberculosis*

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Abstract

Cytokine gene expression in ileal tissues of cattle infected with *Mycobacterium paratuberculosis* was evaluated. The effects of infection with *M. paratuberculosis* on cytokine production may influence immune regulation at the site of colonization, resulting in the chronic inflammatory state associated with the latter stages of this disease. Ileal samples were obtained at necropsy from noninfected control cows ($n = 8$) and clinically infected cows ($n = 7$) and processed for immunohistochemistry and in situ hybridization. Cows infected with *M. paratuberculosis* were in the latter stages of disease with clinical signs such as weight loss, watery diarrhea, and inappetence. Among cytokines we studied, interleukin-1α (IL-1α), IL-1β, IL-6, and interferon-γ (IFN-γ) were expressed significantly more in infected animals than in noninfected control animals. The expression of tumor necrosis factor-α (TNF-α), however, was not different between the two groups of cattle. In addition, immunohistochemical staining demonstrated that the number of resident macrophages in the ileum of infected animals was three times greater than that of noninfected cows. In contrast to this, ileal tissues from noninfected control animals contained 1.5 times more neutrophils than the ileal tissues from cows infected with *M. paratuberculosis*. These data demonstrate that localized ileal cytokine production is different between cows chronically infected with *M. paratuberculosis* and noninfected control cows. Published by Elsevier Science B.V.

Keywords: *Mycobacterium paratuberculosis*; Cytokine genes; Cattle

Abbreviations: IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α

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1. Introduction

Paratuberculosis (Johne's disease) is a chronic enteritis in ruminants caused by the intracellular pathogen, *Mycobacterium paratuberculosis* (Cocito et al., 1994; Stabel, 1998). Infection with *M. paratuberculosis* is more likely to occur in young calves, yet there is a long latency period (2–3 years) before clinical signs become apparent. The characteristic signs of clinical disease are weight loss, episodic watery diarrhea, rough hair coat, or death (Cocito et al., 1994; Stabel, 1998). The most recent survey of dairy herds in the US, conducted by the National Animal Health Monitoring System in 1996, estimated that 20–40% of herds had some degree of *M. paratuberculosis* infection (Wells et al., 1998). Using these prevalence data along with management information obtained from producers, Johne's disease was estimated to generate losses of US$ 220 million per year for the US dairy industry (Ott et al., 1999). This figure is extrapolated from financial losses due to culling or death of clinically infected cows, and reduced reproductive efficiency, feed efficiency, and decreased milk production in subclinically infected animals.

The most common route of infection with *M. paratuberculosis* is by ingestion of contaminated fecal material, milk, or colostrum from clinically infected dams. Once ingested, it has been reported that *M. paratuberculosis* enter into the lymphatic system via the M cells, similar to other enteric intracellular pathogens such as salmonella (Momotani et al., 1988). Live microorganisms traverse the M cells and are scavenged by macrophages or dendritic cells on the basolateral side of the cell where the bacteria may degraded and processed by the phagocytes. However, pathogenic mycobacteria are relatively resistant to the degradative mechanisms of macrophages. In fact, virulent mycobacteria may survive and multiply within macrophages rather than be killed (Laneelle and Daffe, 1991). It is suggested that mycobacteria can survive inside of macrophages by disarming several defense mechanisms such as elevated synthesis of stress proteins, oxygen radicals, disruption of phagosomal membranes as well as inhibition of macrophage priming and activation by cytokines (Laneelle and Daffe, 1991; Rastogi, 1990). During incubation periods lasting several years extensive granulomatous inflammation occurs in the terminal ileum, leading to malabsorption and protein-losing enteropathy. The role of cytokines in the regulation of mycobacterial infection is very complicated and not clearly understood, particularly at the site of tissue colonization. Little information is available concerning the expression of cytokines at the site of ileal inflammation in *M. paratuberculosis*-infected cattle. In the present study, we utilized in situ hybridization to determine effects of *M. paratuberculosis* infection on the expression of various cytokine genes as markers of macrophage activation in bovine ileal tissue.

2. Materials and methods

2.1. Tissue collection and preparation

Fifteen Holstein cattle were used for this experiment. The noninfected control cows ($n = 8$) were characterized by repeated negative fecal cultures performed quarterly over a 3–5-year period. In addition, these animals were negative on any serologic assays (ELISA,

IFN-γ) performed during that period. Positive cows were classified as clinically infected according to a high level of shedding (>100 cfu/g) of *M. paratuberculosis* in their feces and clinical signs of disease such as intermittent bouts of diarrhea, rapid weight loss and inappetence in the immediate period prior to obtaining the tissue samples. Prior to necropsy, animals were sedated with an intravenous injection of xylazine (0.1 mg/kg), and euthanized with an overdose of pentobarbital. Ileal samples of all cattle for immunohistochemical staining and in situ hybridization were collected at necropsy by removing the first 6 in. of the ileum beginning at the ileocecal junction and promptly fixing a 1 in. section in neutral buffered 10% zinc formalin for histologic preparation. Sections of ileal tissue were processed routinely and embedded in paraffin wax. Sections were cut at 6 μm and stained with hematoxylin and eosin (HE) and Ziehl Neelsen (ZN) by conventional methods. Replicate unstained sections were processed for immunohistochemistry and in situ hybridization. Ileal tissues from seven clinically infected cows stained positive by ZN indicating the presence of high numbers of acid-fast bacilli within the cytoplasm of macrophages. The macrophages were present in the lamina propria and submucosa most frequently. Tissues from noninfected control cows were negative for acid-fast bacilli.

2.2. Probe synthesis for in situ hybridization

Bovine sequence-specific RNA probes for five different cytokines, interleukin-1α (IL-1α), IL-1β, IL-6, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and a housekeeping gene, β-actin (β-Act) were synthesized. Briefly, cDNA was reverse transcribed from bovine leukocyte total RNA using random hexameric primers and subjected to PCR amplification using probe-specific primer pairs (Table 1). The amplified DNA fragments of 156, 180, 201, 250, 119 and 80 bp for IL-1α, IL-1β, IL-6, IFN-γ, TNF-α and β-Act, respectively, were cloned into a pGEM4 vector (Promega, Madison, WI), transformed into *Escherichia coli* strain JM109, and their sequences were confirmed by dideoxy sequencing.

Table 1
Sequence of oligomers used for polymerase chain reaction amplification of bovine cytokine-specific cDNA fragments used for synthesis of riboprobes

Cytokine gene	Primer sequence
IL-1α forward primer	5′-GCGAATTCACAGCAGTTGGAATAAGCCGTG-3′
IL-1α backward primer	5′-CCAAGCTTGCACAGAGTTGGACATGACTGAAG-3′
IL-1β forward primer	5′-GCGAATTCAAGGCTCTCCACCTCCTCTC-3′
IL-1β backward primer	5′-CCAAGCTTGTCTACTTCCTCCAGATGCA-3′
IL-6 forward primer	5′-GCGAATTCTGGGTTCAATCAGGCGATTTG-3′
IL-6 backward primer	5′-CCAAGCTTAGGTCAGTGTTTGTGGCTGGAGTG-3′
IFN-γ forward primer	5′-GCGAATTCTTACTGCTCTGTGGGCTTTTGG-3′
IFN-γ backward primer	5′-CCAAGCTTTGCTCCTTTGAATGACCTGGTTATC-3′
TNF-α forward primer	5′-GCGAATTCAAGCCCTGGTACGAACCCATCTAC-3′
TNF-α backward primer	5′-CCAAGCTTTAGACCTGCCCAGACTCAGCATAG-3′
β-Act forward primer	5′-GCGAATTCCTGTCCACCTTCCAGCAGATGT-3′
β-Act backward primer	5′-CCAAGCTTTTCGAAAACGCCACCTGTTACC-3′

For riboprobe synthesis, plasmid DNA was isolated and linearized with either *Eco*R1 or *Hind*III restriction endonucleases, for antisense or sense strand probe, respectively. The linear plasmid DNA was transcribed in vitro using either T7 or SP6 RNA polymerase with ATP, CTP, GTP and digoxigenin labeled UTP (Boehringer Mannheim, Indianapolis, IN) as a labeling agent for antisense and sense strand probe, respectively. The antisense strand probe was used for detection of gene expression because it hybridizes with mRNA by forming a complimentary double strand. The sense strand probe was used as a negative control to assess nonspecific binding.

2.3. In situ hybridization

Paraffin-embedded tissue sections were deparaffinized and treated with 10 μg/ml proteinase K (Boehringer Mannheim) for 30 min at 37°C. After washing slides with diethyl pyrocarbonate-treated (DEPC, Sigma, St. Louis, MO) phosphate-buffered saline slides were air-dried. An amount of 50 μl of RNA probe (0.5 mg of RNA/ml) in hybridization solution (50% formamide, 25% DEPC–H_2O, 3 × sodium chloride/sodium citrate (SSC), 1 × Denhardt's solution, 0.2 mg/ml yeast tRNA, 50 mM sodium phosphate, pH 7.4 and 10% dextran sulfate) was applied onto a sample slide and covered with a coverslip. Slides were heated at 90°C for 10 min and were hybridized for 16 h in a humidified chamber at 60°C. To remove unbound probe, slides were incubated for 30 min at 37°C with RNase A (20 mg/ml, Boehringer Mannheim), washed sequentially in 2 × SSC, 1 × SSC for 5 min each and 0.5 × SSC for 1 h at 60°C, and finally with 0.5 × SSC for 5 min at room temperature. Slides were then incubated with sheep anti-digoxigenin antibody labeled with alkaline phosphatase and developed in 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium (NBT/BCIP, Boehringer Mannheim) solution in the dark for 18 h. After chromogen development, slides were counter-stained with nuclear fast red for 3 min and coverslips were mounted with aqueous mounting media (Accurate Chemical & Scientific Corp., Westbury, NY) (Angerer et al., 1987).

2.4. Macrophage staining

Monoclonal antibody EBM11 (anti-CD68, DAKO, Carpenteria, CA) was used for detection of macrophages in ileal tissues (Ackermann et al., 1994). Briefly, sections of ileal tissues were deparaffinized and treated with 0.25% bacterial protease (Type XIV; Sigma) in Tris buffer (pH 7.6) for 30 min at 37°C. The primary antibody, monoclonal mouse anti-CD68, was diluted 1:25, applied and incubated overnight at 4°C. A peroxidase-labeled goat anti-mouse IgG was used as the secondary antibody, followed by 3′,3-diaminobenzidine as the substrate (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Shandon's hematoxylin was used for counterstaining.

2.5. Neutrophil staining

For the neutrophil staining, lead thiocyanate (Pb(II) SCN, Aldrich, Milwaukee, WI) was used for antigen retrieval in this experiment (Lee et al., 2000). Deparaffinized tissues were treated in boiling Pb(II) SCN (1%) for 1.5 min, then remained in the same solution an

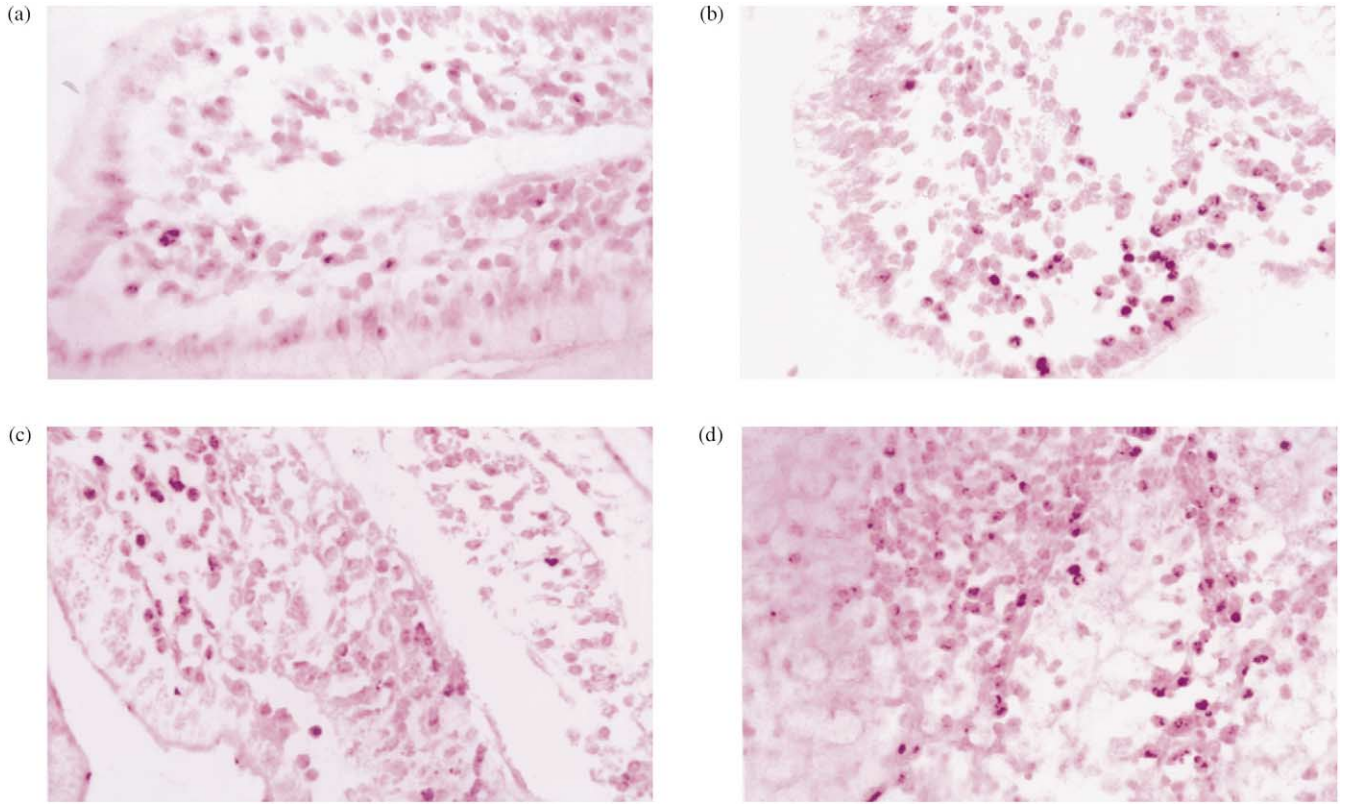

Fig. 1. Photomicrographs of bovine ileal tissue in which IL-1α or IFN-γ mRNA is detected by in situ hybridization. Hybridized cells were stained with alkaline phosphatase and NBT/BCIP substrate (magnification = 1000×): (a) noninfected control cow examined for IL-1α; (b) *M. paratuberculosis*-infected cow examined for IL-1α; (c) noninfected control cow examined for IFN-γ; (d) *M. paratuberculosis*-infected cow examined for IFN-γ.

additional 10 min with the heat turned off (beaker still on heating block). Slides were allowed to cool for 15 min at room temperature in the saturated Pb(II) SCN solution under a hood. Endogenous peroxidase blocking was performed by submerging sections in methanol supplemented with 0.3% hydrogen peroxide for 20 min at room temperature. The sections were treated with 10% normal goat serum for 15 min and labeled with 1:150 dilution of anti-mouse IgG (Fab-specific) biotin conjugated antibody (Sigma, St. Louis, MO) for 1 h at room temperature. The sections were then incubated with streptavidin-horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 30 min and subjected to a color reaction with diaminobenzidine (DAB, Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 15 min. After 15 min incubation, the sections were counterstained with hematoxylin for 1 min and coverslipped. Neutrophils were verified by their characteristic morphologic features (hypersegmented nucleus with pale to neutral-staining cytoplasm) and stained cells were not counted unless they had this characteristic morphology.

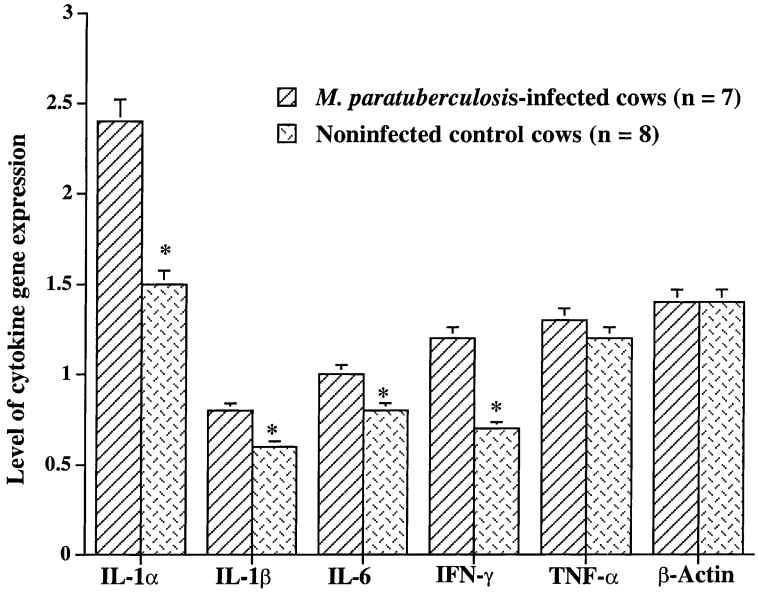

Fig. 2. Differences in proinflammatory cytokine gene expression in ileal tissues from *M. paratuberculosis*-infected and noninfected control cows. Level of cytokine gene expression means the intensity of staining per cell. Subjective scoring was applied according to color reaction — 0: no signal; 1: low expression; 2: moderate expression; 3: high expression. The scores represent the average of an individual tissue within a group with standard error of the mean. For each tissue, five randomly assigned areas of the tissue were chosen and 100 cells were assessed within each area. The data were examined using the Kruskal–Wallis one-way analysis of variance by ranks to evaluate the effect of *M. paratuberculosis* infection on the expression of cytokine genes. The asterisk indicates a statistically significant difference ($P < 0.05$) between *M. paratuberculosis*-infected and noninfected control cows within a group for expression of a given cytokine.

2.6. Data analysis

Two scoring systems were applied to five independent fields on each slide as previously described (Lee et al., 2000). A slide represented an ileal tissue sample from each animal within the two groups. In each of the five fields of a given slide, 100 cells were assessed for evaluation. The mean of each slide was calculated from five independent observations in a given tissue sample. The mean of a given group was expressed as an average of the individual tissue means for all animals within a treatment group, with standard error of the mean. The first assessment was to evaluate the level of gene expression on a per cell basis. Subjective scoring of cytokine gene expression was scaled according to the color reaction — 0: no chromogen staining; 1: pale blue; 2: blue to purple; 3: dark purple. Another criterion for the scoring system was the percentage of positive cells identified in the first evaluation. Subjective scoring of positive cells was as follows — 0: no positive cells; 1: <30% of positive cells; 2: 30–70% of positive cells; 3: >70% of positive cells. A scoring system was used to determine mean values for statistical analysis to normalize the variability between animals within a treatment group. The mean of each slide was

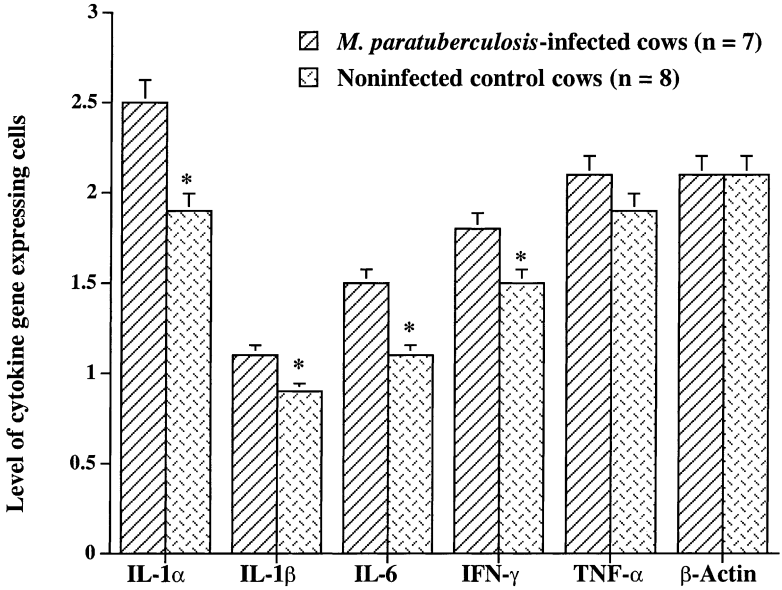

Fig. 3. Amount of cells that expressed proinflammatory cytokine genes in ileal tissues from *M. paratuberculosis*-infected and noninfected control cows. Values for cytokine expression-positive cells are estimates of the percentage of cells that hybridized with a given cytokine RNA probe. Subjective scoring was used for estimation of positive cells — 0: no positive cells; 1: <30% of positive cells; 2: 30–70% of positive cells; 3: >70% of positive cells. Scores represent the average for each tissue within a group with standard error of the mean. The data were examined using the Kruskal–Wallis one-way analysis of variance by ranks to evaluate the effect of *M. paratuberculosis* infection on expression of the cytokine genes. The asterisk indicates a statistically significant difference ($P < 0.05$) between *M. paratuberculosis*-infected and noninfected control cows within a group for expression of a given cytokine.

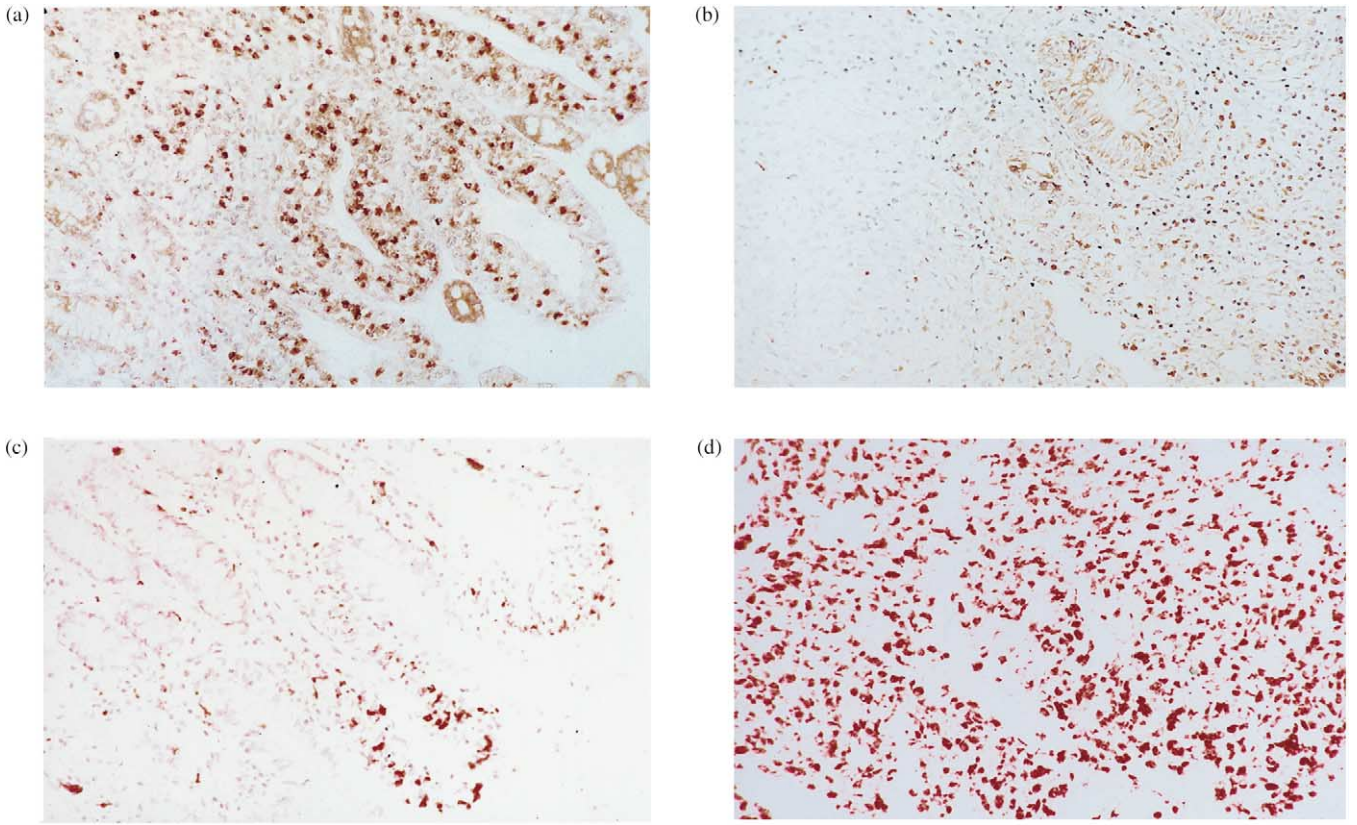

Fig. 4. Photomicrographs of bovine ileal tissues stained with either monoclonal mouse anti-CD68 for macrophages or goat anti-mouse IgG (Fab-specific) for neutrophils (magnification = 100×): (a) ileal tissue from noninfected control cow examined for neutrophils; (b) ileal tissue from *M. paratuberculosis*-infected cow examined for neutrophils; (c) ileal tissue from noninfected control cow examined for macrophages; (d) ileal tissue from *M. paratuberculosis*-infected cow examined for macrophages.

calculated from scoring performed on five independent field of 100 cells each for a given tissue sample. The mean of a given group was an average of the individual tissue means for all animals within a treatment group, with standard error of mean.

The data were examined using the Kruskal–Wallis one-way analysis of variance by ranks to evaluate the effects of *M. paratuberculosis* infection on the expression of cytokine genes.

3. Results

Results from this study indicate that infection with *M. paratuberculosis* induced cytokine gene expression and increased the number of cells expressing the cytokine genes in ileal tissue compared to noninfected control cows (Figs. 1 and 2). The induction of IL-1α, IL-1β, IL-6 and IFN-γ gene expression was significantly ($P < 0.05$) greater for infected cattle, with the most dramatic differences noted for IL-1α and IFN-γ (Fig. 2). The same pattern was observed for the number of cells that expressed given cytokine genes within a tissue sample (Fig. 3). In this regard, IL-1α was again the most prominent cytokine, as a greater ($P < 0.05$) number of cells in the ileum of *M. paratuberculosis*-infected cattle expressed IL-1α than cells in the ileum of noninfected control cattle. IFN-γ and IL-6 were also expressed in more ($P < 0.05$) cells of infected cattle than noninfected animals. There was no significant difference in TNF-α gene expression between the infected and noninfected treatment groups (Figs. 2 and 3).

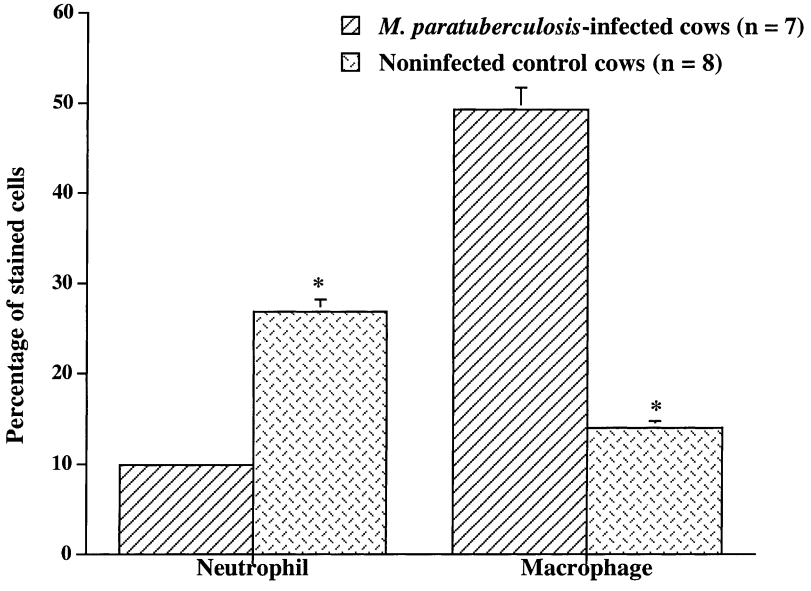

Fig. 5. Percentage of labeled neutrophils and macrophages in ileal tissues of *M. paratuberculosis*-infected and noninfected control cows.

Significant differences were observed in the number of phagocytes present in ileal tissue in cows infected with *M. paratuberculosis* compared to noninfected controls (Fig. 4). The macrophage stained most predominantly in the ileum of infected animals, with only negligible staining of neutrophils noted (Fig. 5). In contrast, neutrophils were the predominant phagocytic cell in the ileum of control cattle with significantly ($P < 0.05$) lower numbers noted for cows infected with *M. paratuberculosis*.

4. Discussion

In the present study, infection with *M. paratuberculosis* substantially increased expression of IL-1α, IL-1β, IL-6, and IFN-γ genes in sections of ileal tissues taken from clinically infected cows. Mycobacterial infection tends to be of a chronic nature with long periods of latency, regardless of mycobacterial species. The latency periods associated with mycobacterial disorders can be attributed to the ability of these intracellular pathogens to survive within macrophages of the infected hosts. The host immune responses to mycobacterial infections in general are paradoxical with a reciprocal relationship between T cell responsiveness and extent of disease as observed in patients with tuberculosis or leprosy (Orme et al., 1993; Yamamura et al., 1991). Mycobacteriostatic effects in the early stages of disease appear to be mediated by CD4+ T cells and their cytokine products. These cytokines are important activators of bacteriocidal and bacteriostatic activity of macrophages (Cocito et al., 1994; Mutis et al., 1993; Sussman and Wadee, 1992).

Chronic inflammation of the small intestine (primarily the ileum and jejunum) is a hallmark characteristic of *M. paratuberculosis* infection, particularly in the latter stages of disease. Therefore, this study was designed to evaluate the effects of clinical disease on the expression of proinflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α at the site of tissue colonization. We also evaluated effects of clinical infection on expression of IFN-γ since this is an important activator of macrophages and has been eschewed as the most critical cytokine for controlling mycobacterial infections. Among the cytokine genes that we evaluated, increased expression of IL-1α and IFN-γ genes in the ileal tissue of cattle infected with *M. paratuberculosis* was the most pronounced. Expression of IL-1α may be significant since it has been observed that monocytes from cattle naturally infected with *M. paratuberculosis* constitutively secreted more than 10 times the amount of IL-1 in comparison to cells from noninfected cattle (Kreeger et al., 1991). IL-1 plays a major role in the activation of T cells and clonal expansion of T cell subsets so increased expression of this gene in tissue from infected cows would seem logical since host macrophages should be highly activated due to their bacterial load. Yet, the presence of IL-1 in the tissue may be more adversarial in nature since it has been reported that IL-1α can deactivate macrophages and thereby enhance intracellular growth of mycobacteria (Wallis et al., 1992).

More surprising was the negligible effect of *M. paratuberculosis* infection on expression of TNF-α, a major proinflammatory cytokine, in ileal tissues. However, it has been shown that monocytes from patients with chronic tuberculosis usually produce less TNF compared to the monocytes of healthy individuals (Takashima et al., 1990). Ileal tissues used in this study were obtained from clinically infected cows in the latter stages of disease

and TNF-α expression may have been downregulated in these animals compared to a more acute inflammatory state observed in other bacterial diseases. It has also been noted that IL-6 secreted in mycobacterial infection may antagonize the function of TNF-α (Bermudez et al., 1992; Blanchard et al., 1991) and we did observe increased expression of IL-6 in ileal tissue of infected cows. Cytokines may be both beneficial and detrimental to the host, depending upon the amount of cytokine secreted and its subsequent effects on regulation of expression of other cytokines that influence cell activation. At high doses (1000 and 4000 IU/ml) of rTNF-α, macrophages suppressed the intracellular growth of *M. paratuberculosis* but at moderate doses (10 and 100 IU/ml) of TNF, the bacilli actually survived and replicated within the cell (Stabel, 1995).

Studies evaluating other mycobacterial species such as *M. tuberculosis* have shown that CD4+, CD8+ and γδ T cells are key cell populations involved in resistance to infection and that IFN-γ is the critical cytokine required for cellular activation (Cooper and Flynn, 1995). This observation would suggest that IFN-γ production would be decreased during the latter stages of *M. paratuberculosis* infection when the host animal is no longer able to control the spread of infection and clinical signs become apparent. Indeed, Sweeney et al. (1998) observed that IFN-γ expression in ileal tissue and cecal lymph nodes was greater in cows with subclinical paratuberculosis compared to cows with clinical disease, whereas similar levels of gene expression were noted between clinically infected cows and noninfected cows. In the present study we observed increased gene expression of IFN-γ in the ileal tissue of cows with clinical infection. Higher IFN-γ secretion was noted by peripheral blood mononuclear cells (PBMC) isolated from naturally infected cows compared to control noninfected cows after stimulation of cells with either concanavalin A, a T cell mitogen, or a whole cell sonicate of *M. paratuberculosis* (Stabel, 1996). In a human study, PBMC from healthy individuals or patients with tuberculoid leprosy or lepromatous leprosy all produced Th1 type cytokines including IFN-γ when stimulated with a variety of mycobacteria (Mutis et al., 1993). Usually, Th1 cells function to prime or activate phagocytic cells by producing immunologically potent cytokines (Hoffner and Svenson, 1991). One explanation for the higher IFN-γ expression is that T cell derived IFN-γ may be priming macrophages so that they are able to secrete higher amounts of chemotatic molecules thereby provoking further accumulation of cells at the site of infection (Flesch and Kaufmann, 1990, 1993). This may explain the extremely high number of macrophages noted in the ileum of infected cattle in this experiment.

In conclusion, we detected higher levels of expression of IL-1α, IL-1β, IL-6 and IFN-γ in the ileal tissues of *M. paratuberculosis* infected cattle. Although cattle were in the clinical stages of disease when tissues were taken, the proinflammatory cytokine, TNF-α, was not induced by the infection with the bacteria. Expression of IL-1α, IL-1β and IL-6 would indicate that macrophages were highly activated during clinical disease. In addition, higher IFN-γ gene expression noted for the clinically infected cows would suggest that localized T cells were being primed by macrophages and were capable of producing a cytokine which should further activate macrophage mycobactericidal activity. However, the flagrant inflammatory state and high bacterial load in the ileal tissue of the infected cows would suggest a dysfunction in the signaling between macrophages and T cells in the clinical stage of disease. The effects of *M. paratuberculosis* infection on the expression of specific cytokines may explain, in part, the

resulting chronicity of infection and failure of these animals to recover once they succumb to the multibacillary stage of disease.

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